# Crucial role of AIM/CD5L in the development of glomerular inflammation in IgA nephropathy

Running title: AIM/CD5L in IgA nephropathy

Akiko Takahata[1], Satoko Arai[2], Emiri Hiramoto[2], Kento Kitada[2,3], Rina Kato[1], Yuko Makita[1], Hitoshi Suzuki[1], Junichiro Nakata[1], Kimi Araki[4], Toru Miyazaki[2*] and Yusuke Suzuki[1*]

[1] Department of Nephrology, Juntendo University Faculty of Medicine, Tokyo, Japan

[2] Laboratory of Molecular Biomedicine for Pathogenesis, Center for Disease Biology and Integrative Medicine, Faculty of Medicine, The University of Tokyo, Tokyo, Japan

[3] Cardiovascular and Metabolic Disorders, Duke-NUS Medical School, Singapore

[4] Institute of Resource Development and Analysis, Division of Developmental Genetics, Kumamoto University, Kumamoto, Japan

Word count for abstract: 249

Word count for text: 3153

*Correspondence should be addressed to both professors below:

Yusuke Suzuki, M.D. Ph.D

Department of Nephrology, Juntendo University Faculty of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, Japan, +81-3-5802-1064, yusuke@juntendo.ac.jp

Toru Miyazaki, M.D. Ph.D

Laboratory of Molecular Biomedicine for Pathogenesis, Center for Disease Biology and Integrative Medicine, Faculty of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan, +81-3-5841-1436, tm@m.u-tokyo.ac.jp

**Key words**

apoptosis inhibitor of macrophage, IgA nephropathy, macrophage

**Significance statement**

Apoptosis inhibitor of macrophage (AIM) is involved in various diseases. It aids in acute kidney injury recovery by removal of dead cells in urinary tubules. Moreover, feline renal failure is due to dysfunctional AIM scavenging in macrophages. This study aimed to clarify the role of AIM in IgA nephropathy model mice. AIM-deficient IgA nephropathy (IgAN) model mice (*AIM*$^{-/-}$gddY mice) showed IgA deposition similar to that of wild-type gddY mice but lacked glomerular accumulation of IgM/IgG/complement and subsequent regional inflammation, avoiding glomerular sclerosis, proteinuria, and hematuria. The IgAN phenotype was restored with IgM/IgG-IgA immune complex formed by recombinant AIM. This is the first study to elucidate the role of AIM in IgAN, which may facilitate development of new IgAN therapies.

**Abstract**

*Background* IgA nephropathy (IgAN) is initiated by aberrant IgA deposition in glomeruli, followed by IgM/IgG/complement co-depositions, resulting in chronic inflammation and glomerular damage. However, the mechanism that drives such phlogogenic cascade has been unclear. Recently, apoptosis inhibitor of macrophage (AIM) was shown to modulate macrophages' function in various pathological conditions, thereby profoundly affecting the progression of renal disorders, including acute kidney injury. Here, using a spontaneous IgAN model, grouped ddY (gddY) mouse, we examined whether AIM is requisite for the overall inflammatory glomerular injury following IgA deposition.

*Methods* We established an AIM-deficient IgAN model (*AIM*$^{-/-}$gddY) by CRISPR/Cas9 system and analyzed its phenotype compared to that of wild-type gddY with or without recombinant AIM administration. An IgA-deficient IgAN model (*IgA*$^{-/-}$gddY) was also generated to further determine the role of AIM.

*Results* In both human and murine IgAN, AIM colocalized with IgA/IgM/IgG in glomeruli, whereas no AIM deposition was observed in control kidneys. Although *AIM*$^{-/-}$gddY showed IgA deposition at levels comparable to those of wild-type gddY, they lacked glomerular accumulation of IgM/IgG complements, CD45$^{+}$ leukocyte infiltration and up-regulation of

inflammatory/fibrogenic genes; thus protected from glomerular lesions and proteinuria/hematuria. Such IgAN phenotype was reconstituted by recombinant AIM administration, resulting in IgM/IgG/complement-IgA co-deposition. Neither spontaneous IgM/IgG co-depositions nor disease was observed in *IgA*$^{-/-}$gddY.

*Conclusions* AIM may contribute to a stable immune complex formation in glomeruli, thereby facilitating IgAN progression, suggesting that the AIM deposition blockage or disassociation from IgM/IgG serves as a new therapeutic target owing to its role in IgAN inflammatory initiation.

## Introduction

IgA nephropathy (IgAN) was first described more than 50 years ago and is the most common causative disease of primary chronic glomerulonephritis worldwide. It has a poor prognosis, and 30%–40% of patients with IgAN progress to end-stage renal failure within 20 years of diagnosis.[1-3] One of the most important histologic characteristics broadly observed in the kidney of patients with IgAN is the deposition of polymeric IgA1 that forms an immune complex together with variable IgG and IgM, as well as complement 3 (C3) at the glomerular mesangial region.[4-9] Immunodeposits are presumed to cause persistent inflammatory immune responses, resulting in the proliferation of mesangial cells, macrophage infiltration, and matrix expansion, leading to further glomerular damage. The mechanisms underlying the development of the disease after IgA deposition are not yet fully understood, and no effective disease-specific treatments are established.

The grouped ddY (gddY) mouse is one of the best animal models for human IgAN. We previously established the gddY model using ddY mice that spontaneously exhibited mild IgAN-like clinical symptoms, using selective intercrossing for more than 20 generations.[10] All gddY mice develop proteinuria by 8 weeks of age, followed by glomerular sclerosis and obvious renal failure with elevated serum creatinine (Cre) levels by 24 weeks of age. Human

IgAN patients and gddY mice share many pathological features, including glomerular deposits of an IgA-containing immune complex. Macrophages/monocytes are also known to be involved in IgAN pathogenesis.[11, 12] We ascertained this in gddY mice, in which we found $Gr1^{-}$ and $CD115^{+}$ monocytosis in the peripheral blood. Administration of an anti-APRIL neutralization antibody, which reduced glomerular IgA deposition, could suppress monocytosis, confirming the involvement of macrophages/monocytes in IgAN progression.[13, 14]

An apoptosis inhibitor of macrophage (AIM/CD5L, encoded by the *cd5l* gene) is produced by resident tissue macrophages and was initially identified as an apoptosis inhibitor that supports the survival of macrophages against apoptosis-inducing stimuli.[15] It is involved in various diseases, including arteriosclerosis, dyslipidemia, and autoimmune disorders, and acts by regulation of macrophages. [16-20] In the blood, AIM associates with IgM pentamers, which protect it from renal excretion and maintain high levels of circulating AIM (approximately 5 μg /mL in humans and 2 μg /mL in mice).[16] Recently, we clarified the method by which AIM binds to an IgM pentamer through single-particle negative-stain electron microscopy for direct observation of the complex. We found that the *bona fide* shape of IgM pentamer is a hexagon with one piece missing, wherein AIM stably associates via a disulfide bond and a charge-based interaction.[21] Interestingly, during acute kidney injury (AKI), AIM dissociates from IgM pentamers in the blood. The IgM-free AIM passes through the glomerular membrane and

accumulates on AKI-associated intraluminal dead cell debris that obstructs renal proximal tubules. Deposited AIM then promotes phagocytic removal of the debris, contributing to overall kidney tissue repair.[22]

The involvement of AIM in kidney diseases via macrophages as well as its affinity for IgM prompted investigation into its possible roles in IgAN pathogenesis. The purpose of this study was to elucidate the role of AIM in the inflammatory cascade underlying IgAN.

## Methods

### Mice

The gddY mice were established by selective mating of early-onset ddY mice for more than 20 generations.[10] AIM-deficient and IgA-deficient gddY mice were generated using the CRISPR/Cas9 system.[23] The plasmid pX330 (Addgene, Watertown, MA, USA) with the guide sequence of the target gene inserted, were microinjected into fertilized embryos of gddY mice. Mutations in the gene of interest were confirmed by examining DNA sequences of PCR-amplified fragments that contained the targeted area sequence. Homozygous knockout mice were obtained by intercrossing of heterozygous mice. Therefore, 23 base pairs including the start codon and 7 base pairs including the beginning of the Cα region were deleted in AIM-deficient and IgA-deficient gddY mice, respectively. AIM and IgA were absent from serum samples of the respective homozygous mice, as confirmed by Western blotting.

BALB/c mice were purchased from Sankyo Labo Service Corporation Inc. All mice were maintained at the animal facility of Juntendo University on a regular chow (Oriental Yeast, Tokyo, Japan) and water ad libitum in a specific-pathogen-free room. The experimental protocol of this study was approved by the Ethics Review Committee for Animal Experimentation of Juntendo University Faculty of Medicine (permit number: 270110).

**Human subjects**

Renal biopsy specimens were obtained at Juntendo University Hospital with the informed consent from patients and approval of the Research Ethics Review Committees of Juntendo University Hospital.

**Generation of anti-AIM antibodies**

We obtained a recombinant baculovirus encoding AIM protein plus 6xHistidine-Tag by homologous recombination with the pBlueBac4.5 transfer vector (V1995-20; Invitrogen, Waltham, MA, USA) into which we subcloned an AIM cDNA fragment encoding the entire open reading frame. We transfected the recombinant baculovirus into egg cells of *Trichoplusia* (HighFive cells, B855-02; Invitrogen), and we purified rAIM protein from the conditioned medium using a histidine-tag affinity column. We immunized rabbits four times with the purified AIM protein.[15] Polyclonal anti-AIM serum has been used for many years in many publications.[16-18, 22] Its specificity was fully confirmed by the fact that this antibody does not stain tissues and blood collected from AIM-deficient mice either by immunohistochemistry or

by immunoblotting. As demonstrated in Figure S1, the antibody did not crossreact with IgA, IgG, and IgM, and the antibody showed no recognition of any type of immunoglobulin.

**Antibodies and reagents**

Antibodies and reagents used for histological and biochemical analyses were as follows: primary antibodies included goat anti-mouse IgG (ab175661; Abcam, Cambridge, UK), goat anti-mouse IgA (ab97013; Abcam), goat anti-mouse IgM (ab175702; Abcam), horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG-Fc antibody (A90-131P; Bethyl Laboratories, Montgomery, AL, USA), HRP-conjugated goat anti-mouse IgM (A80-100P; Bethyl Laboratories), HRP-conjugated goat anti-mouse IgA (14-18-0; KPL, Milford, MA, USA), rabbit anti-AIM (rab2 rabbit polyclonal for mice and human, customized in our laboratory), rat anti-mouse C3 (SC58926; Santa Cruz, Dallas, TX, USA), rabbit anti-mouse C5b9 (bs-2673R-A488; Bioss, Woburn, MA, USA), rat anti-mouse CD45 (550539; BD Pharmingen, Franklin Lakes, NJ, USA), rabbit anti-human IgA (F0316; Dako, Santa Clara, CA, USA), goat anti-human IgM (218; MBL, Nagoya, Japan), HRP-conjugated goat anti-human IgM (A80-100P, Bethyl Laboratories), HRP conjugated with goat anti-rabbit IgG (32460; Thermo Fisher Scientific, Waltham, MA, USA), goat anti-human IgG (109-476-098; Jackson,

West Grove, PA, USA), goat anti-human IgM (109-476-098; Jackson), rabbit anti-human C3 (F0201, Dako), and rabbit anti-human C5b-9 (bs-2673R-A488; Bioss). Secondary antibodies and related reagents included Alexa 488-conjugated anti-rabbit IgG (A21206; Invitrogen), Alexa 488-conjugated anti-rat IgG (A11006; Invitrogen), Alexa 555-conjugated anti-rabbit IgG (A31572; Invitrogen), Alexa 555-conjugated anti-rat IgG (A21434; Invitrogen), HRP-conjugated goat anti-rabbit IgG (32460; Thermo Fisher Scientific), blocking solution (customized in our laboratory; 0.2% fish gelatin, 2% bovine serum albumin with phosphate-buffered saline [PBS]), and Fluoromount (K024; Diagnostic Biosystems, Pleasanton, CA, USA). Specimens were analyzed using confocal laser microscopy (FV-1000; Olympus Corporation, Tokyo, Japan).

Evaluation of proteinuria and serum creatinine and blood urea nitrogen (BUN) levels

Urinary albumin was measured by immunoassay (DCA 2000 system; SIEMENS Healthcare Diagnostics, Erlagen, Germany). Hematuria was examined by Uro-lab sticks SGL (2321, SIEMENS Healthcare Diagnostics). Serum creatinine and BUN levels were measured using an auto analyzer (Fuji Dry-Chem 5500; Fujifilm, Tokyo, Japan).

**$AIM^{-/-}$gddY analysis and recombinant AIM (rAIM) injection model**

Serum, urine, and kidney samples were collected from *AIM*$^{-/-}$gddY, and the phenotype of the disease was compared with that in gddY mice. rAIM was purified as previously described[22] and injected into the tail vein of *AIM*$^{-/-}$gddY mice; the phenotypes were then analyzed.

**Histological analysis**

For light microscopy, renal specimens were fixed in 15% formaldehyde and embedded in paraffin; 3-μm sections were then collected. Tissue sections were stained with periodic acid–Schiff and Azan stains. For the quantitative analysis of glomerular sclerosis, 20 glomeruli per cross-section were observed, and the following scores were assigned, using a modified method based on the report by Raij et al[24]: 0 points, no glomerular sclerosis; 1 point, mild glomerular sclerosis (approximately 25%); 2 points, moderate glomerular sclerosis (approximately < 50%); and 3 points, severe glomerular sclerosis (approximately >50%). Sclerosis scores were calculated as follows: [Σ (each score × number of glomeruli)]/20.

For fluorescent immunohistochemistry (mouse and human), renal specimens were mounted in OCT compound (4583, Sakura Fine Tek, Tokyo, Japan) and stored at −80°C. Specimens were sliced in 4-μm sections and fixed with acetone at −20°C for 4 min. Slides were blocked in a blocking solution (customized in our laboratory, described earlier in this paper) and then

incubated with a primary antibody for 1 hour at room temperature or overnight at 4°C; slides were subsequently incubated with an appropriate fluorophore-conjugated secondary antibody for 30 min at room temperature. The specimens were subjected to analysis using confocal microscopy (FV10, Olympus). For quantitative analysis of CD45-positive cells, the numbers of positive cells were counted for 15 glomeruli in 8- and 16-week-old gddY and *AIM*$^{-/-}$gddY mice, and the average number of positive cells per glomerulus was analyzed (n = 5).

**Quantitative PCR assay**

Glomeruli were isolated by the differential sieving method.[25] RNA was isolated using the QIAgen RNeasy kit (74104, Qiagen, Düsseldorf, Germany), in accordance with the manufacturer's specifications. cDNA was generated using Taqman Fast Advanced Master Mix (4444556, Thermo Fisher Scientific). TaqMan probes were used for measurement of the expression of cd5l/Aim (Mm00437567_m1), IL-1β (Mm00434228_m1), IL-6 (Mm00446190_m1), Ccl2/MCP-1 (Mm00441242_m1), TNF (Mm00443258_m1), TGF-β (Mm01178820_m1), Acta2/αSMA (Mm01546133_m1), and collagen, type I, alpha 2/COL1α2 (Mm00483888_m1). mRNA expression was adjusted relative to the expression of Gapdh (Mm99999915_g1). All probes were purchased from Life Technologies (Carlsbad, CA, USA).

The quantitative evaluation of mRNA was performed by the ΔΔCT method using a 7500 Fast Real-Time PCR system (Invitrogen).

**Immunoprecipitation**

We coupled rabbit anti-mouse IgA (GTX20289; GENETEX, CA, USA) and rabbit monoclonal anti-human IgA Ab (ACM-ab124716; Abcam) to CNBr-activated sepharose 4B (17043001; GE Healthcare; Chicago, IL, USA), and we immunoprecipitated the serum of each mouse strain, human IgAN, and healthy control. Further, we eluted samples by heating them at 100°C for 5 min with 2× SDS buffer and performed immunoblotting for anti-IgA, anti-IgM, and anti-AIM.

**Statistical analysis**

Results are presented as mean ± standard error of the mean; n defines the number of biologic replicates. Differences between groups were assessed by unpaired two-tailed t-test or two-way ANOVA. P values $< 0.05$ were considered statistically significant. All analyses were performed using Prism software (version 7.0, GraphPad Inc., La Jolla, CA, USA).

## Results

### AIM co-deposited with IgA in murine and human IgAN

In gddY mice, IgA co-deposition with IgG and IgM and subsequent complement activation occurs in glomeruli around 4–8 weeks of age; this results in chronic and persistent inflammation and glomerular lesions.[10] In addition to the involvement of macrophages in the development of IgAN, marked accumulation of AIM was observed at glomeruli, colocalizing with IgA in gddY mice (Figure 1A). AIM deposition in glomeruli was present at multiple ages (multiple stages during the disease process) (Figure S2A). AIM staining in the glomeruli did not correspond to AIM expression by mesangial cells, as no *cd5l/Aim* mRNA was observed in glomeruli isolated from gddY mice but was observed in the liver, which was the positive control. Thus, AIM in the glomerular region was derived from circulating AIM. AIM deposition in glomeruli was rarely detected in BALB/c mice, compared with age-matched gddY mice (Figure 1A). Similar to our observations in gddY mice, AIM accumulation in glomeruli colocalized with IgA in human IgAN patients but was not observed in kidneys from patients with minimal change nephrotic syndrome or membranous nephropathy (Figure 1B).

### No glomerular lesion was observed in *AIM*$^{-/-}$gddY mice

Due to the aberrant AIM deposition in glomeruli in human and murine IgAN, we investigated whether glomerular AIM was involved in IgAN pathogenesis, that is, whether AIM may be required for IgA deposition or may influence the progressive tissue destruction following IgA deposition. To assess this, we directly targeted the *cd5l/Aim* gene in gddY mice by using a CRISPR/Cas9 system. AIM-deficient ($AIM^{-/-}$) gddY mice harbored a 23-base pair deletion around the ATG start codon in the *cd5l/Aim* gene, which resulted in a lack of AIM protein in the serum (Figure S2). At 8 weeks of age, both wild-type (WT) and $AIM^{-/-}$gddY mice showed similar levels of IgA deposition in glomeruli, suggesting that AIM was not necessary for disease-initiating IgA deposition (Figure 1A). However, levels of proteinuria (8 weeks of age, $p < 0.001$; 16 weeks of age, $p < 0.01$), as well as the increased serum levels of Cre and BUN (8 and 16 weeks of age, $p < 0.001$), were significantly lower in $AIM^{-/-}$gddY mice, whereas they were prominent in WT gddY mice (Figure 1C).

**Glomerular IgA deposition did not cause glomerular IgM and IgG co-deposition or complement activation in $AIM^{-/-}$gddY mice**

We next assessed why $AIM^{-/-}$ protected against renal injury, despite glomerular IgA deposition. Surprisingly, immunofluorescent analysis revealed that IgM, IgG, and C3 depositions were

largely absent in *AIM*$^{-/-}$gddY mice (Figure 2A). Accordingly, the development of C5b–9, a membrane attack complex, was apparent in the glomerular region in WT gddY mice but not in *AIM*$^{-/-}$gddY mice, at 8 and 16 weeks of age (Figure 2B, 8 weeks of age, $p < 0.05$; 16 weeks of age, $p < 0.01$). Consistent with these differences in immune responses in the glomerular region, WT gddY mice exhibited obvious glomerular injury by 8 weeks of age, characterized by CD45$^{+}$ leukocyte infiltration ($p < 0.05$), glomerular sclerosis ($p < 0.05$), interstitial fibrosis, mesangial cell proliferation, and extracellular matrix expansion, whereas *AIM*$^{-/-}$ddY mice did not exhibit these pathological phenotypes (Figure 3A and 3B). In accordance with the histologic observations, mRNA levels of inflammatory and fibrogenic genes expressed in glomeruli were significantly lower in *AIM*$^{-/-}$ than in WT gddY mice, when assessed by quantitative PCR (Figure 3C, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). Thus, although IgA deposition occurred as in WT gddY mice, subsequent inflammatory reactions and tissue destruction were markedly prevented in *AIM*$^{-/-}$gddY mice.

**rAIM restored glomerular IgM/IgG co-deposition and glomerular damage in *AIM*$^{-/-}$ mice**

The sole importance of AIM for the completion of the disease was determined by the following experiment. rAIM or PBS control was administered intravenously to *AIM*$^{-/-}$gddY mice (8

weeks old, n = 8 per group), and the glomeruli were analyzed 2 hours later. As shown in Figure 4A, rAIM injection promoted massive accumulation of IgM, IgG, and C3, colocalized with AIM; this was not observed in the PBS control mice. Accordingly, proteinuria and hematuria were also significantly induced (Figure 4B, $p < 0.001$). These findings indicate that glomerular IgA deposition alone does not induce IgAN and that AIM is essential for binding of previously deposited IgA with IgM or IgG, leading to progression of tissue destruction. The co-deposition of AIM with IgM, IgG, C3, and C5b-9 in human IgAN glomeruli also supports our hypothesis (Figures 5A and S4).

**AIM deposition did not occur without glomerular IgA deposition**

Furthermore, we assessed whether the spontaneous glomerular deposition of AIM or IgM/IgG occurred in the absence of IgA, through direct disruption of the *IgA* gene in gddY mice via CRISPR/Cas9 (*IgA*$^{-/-}$gddY). As shown in Figure 5B, no deposition of IgM, IgG, or AIM was observed in the glomeruli of *IgA*$^{-/-}$gddY mice, clearly indicating that aberrant IgA deposition triggers the disease. In addition, it is likely that the formation of an immune complex of IgA, IgM, IgG, and AIM may primarily occur *in situ* because this immune complex with AIM was

not detected in the serum of gddY mice either by immunoprecipitation or by immunoblotting of the IgM fraction (Figures 6 and S5).

## Discussion

IgA with aberrant glycosylation plays an essential role ("1st hit") in the pathogenesis of IgAN. In particular, the levels of an IgA1 variant, which is galactose-deficient in the hinge region (Gd-IgA1), are significantly increased in the blood of patients with IgAN, and the extent of this increase is correlated with disease prognosis.[26-30] However, healthy relatives of patients with IgAN exhibit significantly higher levels of Gd-IgA1 in blood than healthy non-relatives, and autopsy results indicated that glomerular IgA deposits are present in approximately 10%–30% of individuals with normal renal function and no abnormal renal or urinary findings.[31, 32] These findings indicate that increased Gd-IgA1 and glomerular IgA deposition are not entirely responsible for the progression of renal impairment and suggest that factors other than increased Gd-IgA1 deposition underlie disease progression. One factor currently under consideration is the formation of immune complexes of endogenous IgM and IgG autoantibodies that correspond with abnormal glycosylation of IgA. Blood concentrations of immune complexes containing abnormal IgA are likely correlated with the prognosis and severity of human and murine

IgAN.[33] Furthermore, in human IgAN, the co-deposition of glomerular IgM and IgG is correlated with the severity of tissue lesions.[4-8, 32] Although the specific mechanism of immune complex formation has not been previously elucidated, the findings of the present study may provide a critical clue. Firstly, no deposition of IgM, IgG, or AIM was observed in glomeruli of *IgA*$^{-/-}$gddY mice, which indicates that IgA deposition is the initial trigger in IgAN. Secondly, the finding that *AIM*$^{-/-}$gddY mice were free from glomerulonephritis without co-deposition of glomerular IgM and IgG demonstrates that AIM plays a key role in the mechanism underlying formation of nephritogenic IgA and IgM/IgG immune complexes. The restoration of the glomerulonephritis phenotype by administration of rAIM to *AIM*$^{-/-}$gddY also strongly suggests the direct involvement of AIM in immune complex formation.

It is known that AIM stably associates with IgM by fitting into the one missing part of the hexagon in circulating blood in both mice and humans.[21] We also clarified that AIM associates with IgA in blood in both mice and humans (Figures 6 and S7) and particularly binds to IgA-Fc in mice by immunoprecipitation (Figure S6). The binding mechanism of AIM and IgA1 in humans could not be clarified with the same method as in mice, presumably because of aberrant glycosylation in IgA1 and polymeric formation of these IgA1. Thus, we need further evaluation under many different conditions to clarify the binding mechanism in humans in the near future. Although the entire mechanism of how AIM promotes the formation of immune complex

remains unknown, the binding nature of AIM to both IgM[21] and IgA (Figures 6, S6, and S7) suggests the linkage of IgA and IgM via AIM. As previously demonstrated that AIM does not bind to IgG, the association mode of IgG remains unknown. One possibility is that circulating IgG/GdIgA1 immune complexes may directly deposit on glomeruli based on the co-deposition of IgG specific for GdIgA1 in all patients with IgAN, as recently reported by Rizk *et al*.[34] Another possibility is that aberrant IgM accumulation in this region activates the complement cascade, thereby inducing local inflammation. This inflammatory response might recruit IgG, i.e., IgG may colocalize, but not associate, with the AIM/IgA/IgM immune complex.

Our results suggest that the development of IgAN can be divided into two distinct phases: 1) deposition of IgA and 2) formation of the immune complex and complement activation, leading to glomerular damage. The first phase is independent of AIM, whereas AIM is significantly involved in the second phase. In human IgAN patients, the immune complex is presumably generated in the serum in some degree.[35, 36] However, we could not detect complexes of IgA and IgM containing AIM in the sera from IgAN patients; even though we clearly found IgA co-deposition with IgM/IgG and AIM (Figure S7). Hence, the possibility of *in situ* immune complex formation in glomeruli via AIM must be carefully evaluated in the progression of human IgAN.

Our observation that aberrant AIM accumulation induces kidney injury is in agreement with our previous report that unexpected AIM accumulation on hepatocellular carcinoma (HCC) cells causes injury to the cancer cells.[37] This is mainly achieved by activation of the complement cascade, which begins with AIM deposition on HCC cells, thereby suppressing the function of complement inhibitors expressed on the cells through direct binding. Thus, in cancer cells, AIM deposition results in a beneficial outcome (i.e., prevention of HCC), whereas in normal cells, it leads to a detrimental outcome (i.e., initiation of IgAN). However, to the best of our knowledge, AIM does not typically accumulate on normal living cells (Figure 1A and 1B). Hence, undesired pathogenic IgA deposition in glomeruli unexpectedly causes AIM to be identified as a source of pathogenesis during IgAN development.

Structurally, AIM belongs to the scavenger receptor cysteine-rich superfamily and functions as a soluble scavenger receptor. We recently demonstrated AIM deposition on dead epithelial cells obstructing the proximal renal tubules during acute kidney injury; here, AIM facilitates the removal of these cells by enhancing their engulfment by phagocytes, thereby promoting rapid repair of kidney injury.[22] This suggests that AIM may induce rapid clearance of harmful immune complexes or injured mesangial cells, which may exhibit important roles in the repair process. It is certainly possible that AIM has two different activities for IgAN regulation during the initiation (detrimental) and progressive (beneficial) phases. However, as observed in

acute kidney injury, AIM is functionally active when it dissociates from IgM but is inactivated when it associates with the large IgM pentamer. Interestingly, we recently demonstrated that the AIM–IgM binding affinity in cats is 1000-fold higher than that in mice, leading to abrogation of AIM function in cats and subsequent renal failure.[38] Therefore, it is also plausible that AIM is inactive within the immune complex in the glomeruli and does not exert its scavenging function in IgAN. Dissociation of AIM might thus provide a novel therapeutic strategy for IgAN, through both destruction of immune complexes and induction of tissue repair by IgM-free and active AIM. Further studies involving identification of the mechanism by which glomerular IgM/IgG deposition occurs via AIM, as well as how it may be dissociated, could presumably confirm this hypothesis and facilitate the development of new therapies for IgAN.

In conclusion, our data provide new fundamental insights into how glomerular inflammation is triggered after IgA deposition. Our findings suggest that the blockage of AIM deposition or disassociation of AIM from IgM/IgG could serve as a new therapeutic target to take advantage of its role in the inflammatory initiation of IgAN.

**Author contributions**

A.T., S.A., T.M., and Y.S. designed the study; A.T., E.H., K.K., R.K., Y.M., H.S., J.N., and K.A. conducted and analyzed experiments; K.A. established the *AIM*$^{-/-}$gddY and *IgA*$^{-/-}$gddY mice by the CRISPR/Cas 9 method; A.T., S.A., T.M., and Y.S. wrote the manuscript; all authors approved the final version of the manuscript.

**Acknowledgments**

The authors thank Terumi Shibata, Taeko Ikuhara, Yuko Aoki, Aki Omori, Tamami Sakanishi, Akemi Koyanagi, Yuko Kojima, Takako Ikegami and Tomomi Ikeda for their fruitful scientific advice and excellent research assistance.

**Disclosure**

The authors have nothing to declare.

**Funding**

This study was supported by JSPS KAKENHI [Grant Number JP26461237 (to J.N.) and JP16H06389 (to T.M.)] and the AMED-CREST, Japan Agency for Medical Research

Development [Grant number JP18gm0610009 (to T.M.)], and research grants by Kyowa Hakko Kirin Co., Ltd., MSD Co., Ltd. and Boehringer Ingelheim Co., Ltd.

**Supplemental material Table of Contents**

**References**


1. Appel GB, Waldman M: The IgA nephropathy treatment dilemma. *Kidney Int* 69: 1939–1944, 2006.
2. Barratt J, Feehally J: IgA nephropathy. *J Am Soc Nephrol* 16: 2088–2097, 2005.
3. Julian BA, Novak J: IgA nephropathy: an update. *Curr Opin Nephrol Hypertens* 13: 171–179, 2004.
4. Nieuwhof C, Kruytzer M, Frederiks P, van Breda Vriesman PJ: Chronicity index and mesangial IgG deposition are risk factors for hypertension and renal failure in early IgA nephropathy. *Am J Kidney Dis* 31: 962–970, 1998.
5. van Dixhoorn MG, Sato T, Muizert Y, van Gijlswijk-Janssen DJ, De Heer E, Daha MR: Combined glomerular deposition of polymeric rat IgA and IgG aggravates renal inflammation. *Kidney Int* 58: 90–99, 2000.
6. Wada Y, Ogata H, Takeshige Y, Takeshima A, Yoshida N, Yamamoto M, et al.: Clinical significance of IgG deposition in the glomerular mesangial area in patients with IgA nephropathy. *Clin Exp Nephrol* 17: 73–82, 2013.
7. Bellur SS, Troyanov S, Cook HT, Roberts IS, Working Group of International IgA Nephropathy Network and Renal Pathology Society: Immunostaining findings in IgA

nephropathy: correlation with histology and clinical outcome in the Oxford classification patient cohort. *Nephrol Dial Transplant* 26: 2533–2536, 2011.

8. Eison TM, Hastings MC, Moldoveanu Z, Sanders JT, Gaber L, Walker PD, et al.: Association of IgG co-deposition with serum levels of galactose-deficient IgA1 in pediatric IgA nephropathy. *Clin Nephrol* 78: 465–469, 2012.
9. Heybeli C, Unlu M, Yildiz S, Cavdar C, Sarioglu S, Camsari T: IgA nephropathy: association of C4d with clinical and histopathological findings and possible role of IgM. *Ren Fail* 37: 1464–1469, 2015.
10. Okazaki K, Suzuki Y, Otsuji M, Suzuki H, Kihara M, Kajiyama T, et al.: Development of a model of early-onset IgA nephropathy. *J Am Soc Nephrol* 23: 1364–1374, 2012.
11. Bohle A, Wehrmann M, Bogenschutz O, Batz C, Vogl W, Schmitt H, et al.: The long-term prognosis of the primary glomerulonephritides. A morphological and clinical analysis of 1747 cases. *Pathol Res Pract* 188: 908–924, 1992.
12. Cox SN, Sallustio F, Serino G, Loverre A, Pesce F, Gigante M, et al.: Activated innate immunity and the involvement of CX3CR1-fractalkine in promoting hematuria in patients with IgA nephropathy. *Kidney Int* 82: 548–560, 2012.

13. Kim YG, Alvarez M, Suzuki H, Hirose S, Izui S, Tomino Y, et al.: Pathogenic Role of a Proliferation-Inducing Ligand (APRIL) in Murine IgA Nephropathy. *PLoS One* 10: e0137044, 2015.

14. Myette JR, Kano T, Suzuki H, Sloan SE, Szretter KJ, Ramakrishnan B, et al.: A Proliferation Inducing Ligand (APRIL) targeted antibody is a safe and effective treatment of murine IgA nephropathy. *Kidney Int* 96: 104–116, 2019.

15. Miyazaki T, Hirokami Y, Matsuhashi N, Takatsuka H, Naito M: Increased susceptibility of thymocytes to apoptosis in mice lacking AIM, a novel murine macrophage-derived soluble factor belonging to the scavenger receptor cysteine-rich domain superfamily. *J Exp Med* 189: 413–422, 1999.

16. Arai S, Maehara N, Iwamura Y, Honda S, Nakashima K, Kai T,et al.: Obesity-associated autoantibody production requires AIM to retain the immunoglobulin M immune complex on follicular dendritic cells. *Cell Rep* 3: 1187–1198, 2013.

17. Arai S, Shelton JM, Chen M, Bradley MN, Castrillo A, Bookout AL, et al.: A role for the apoptosis inhibitory factor AIM/Spalpha/Api6 in atherosclerosis development. *Cell Metab* 1: 201–213, 2005.

18. Kurokawa J, Arai S, Nakashima K, Nagano H, Nishijima A, Miyata K, et al.: Macrophage-derived AIM is endocytosed into adipocytes and decreases lipid droplets via inhibition of fatty acid synthase activity. *Cell Metab* 11: 479–492, 2010.

19. Kurokawa J, Nagano H, Ohara O, Kubota N, Kadowaki T, Arai S, et al.: Apoptosis inhibitor of macrophage (AIM) is required for obesity-associated recruitment of inflammatory macrophages into adipose tissue. *Proc Natl Acad Sci U S A* 108:12072–12077, 2011.

20. Miyazaki T, Kurokawa J, Arai S: AIMing at Metabolic Syndrome. *Circ J* 75: 2522–2531, 2011.

21. Hiramoto E, Tsutsumi A, Suzuki R, Matsuoka S, Arai S, Kikkawa M, et al.: The IgM pentamer is an asymmetric pentagon with an open groove that binds the AIM protein. *Sci Adv* 4: eaau1199, 2018.

22. Arai S, Kitada K, Yamazaki T, Takai R, Zhang X, Tsugawa Y, et al.: Apoptosis inhibitor of macrophage protein enhances intraluminal debris clearance and ameliorates acute kidney injury in mice. *Nat Med* 22: 183–193, 2016.

23. Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E: A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* 337: 816–821, 2012.

24. Raij L, Azar S, Keane W: Mesangial immune injury, hypertension, and progressive glomerular damage in Dahl rats. *Kidney Int* 26: 137–143, 1984

25. Rops AL, van der Vlag J, Jacobs CW, Dijkman HB, Lensen JF, Wijnhoven TJ, et al.: Isolation and characterization of conditionally immortalized mouse glomerular endothelial cell lines. *Kidney Int* 66: 2193–2201, 2004.

26. Moore JS, Kulhavy R, Tomana M, Moldoveanu Z, Suzuki H, Brown R, et al.: Reactivities of N-acetylgalactosamine-specific lectins with human IgA1 proteins. *Mol Immunol* 44: 2598–2604, 2007.

27. Suzuki H, Moldoveanu Z, Hall S, Brown R, Vu HL, Novak L, et al.: IgA1-secreting cell lines from patients with IgA nephropathy produce aberrantly glycosylated IgA1. *J Clin Invest* 118: 629–639, 2008.

28. Suzuki H, Kiryluk K, Novak J, Moldoveanu Z, Herr AB, Renfrow MB, et al.: The pathophysiology of IgA nephropathy. *J Am Soc Nephrol* 22: 1795–1803, 2011.

29. Berthoux F, Suzuki H, Thibaudin L, Yanagawa H, Maillard N, Mariat C, et al.: Autoantibodies targeting galactose-deficient IgA1 associate with progression of IgA nephropathy. *J Am Soc Nephrol* 23: 1579–1587, 2012.

30. Moldoveanu Z, Wyatt RJ, Lee JY, Tomana M, Julian BA, Mestecky J, et al.: Patients with IgA nephropathy have increased serum galactose-deficient IgA1 levels. *Kidney Int* 71: 1148–1154, 2007.

31. Sinniah R: Occurrence of mesangial IgA and IgM deposits in a control necropsy population. *J Clin Pathol* 36: 276–279, 1983.

32. Suzuki K, Honda K, Tanabe K, Toma H, Nihei N, Yamaguchi Y: Incidence of latent mesangial IgA deposition in renal allograft donors in Japan. *Kidney Int* 63: 2286–2294, 2003.

33. Suzuki Y, Matsuzaki K, Suzuki H, Okazaki K, Yanagawa H, Ieiri N, et al.: Serum levels of galactose-deficient immunoglobulin (Ig) A1 and related immune complex are associated with disease activity of IgA nephropathy. *Clin Exp Nephrol* 18: 770–777, 2014.

34. Rizk DV, Saha MK, Hall S, Novak L, Brown R, Huang ZQ, et al.: Glomerular Immunodeposits of patients with iga nephropathy are enriched for IgG autoantibodies specific for galactose-deficient IgA1. *J Am Soc Nephrol* 30: 2017–2026, 2019.

35. Berthoux F, Suzuki H, Thibaudin L, Yanagawa H, Maillard N, Mariat C, et.al.: Autoantibodies targeting galactose-deficient IgA1 associate with progression of IgA nephropathy. *J Am Soc Nephrol* 23: 1579–1587, 2012.

36. Suzuki H, Fan R, Zhang Z, Brown R, Hall S, Julian BA, et. al.: Aberrantly glycosylated IgA1 in IgA nephropathy patients is recognized by IgG antibodies with restricted heterogeneity. *J Clin Invest* 119: 1668–1677, 2009.

37. Maehara N, Arai S, Mori M, Iwamura Y, Kurokawa J, Kai T, et al.: Circulating AIM prevents hepatocellular carcinoma through complement activation. *Cell Rep* 9: 61–74, 2014.

38. Sugisawa R, Hiramoto E, Matsuoka S, Iwai S, Takai R, Yamazaki T, et al.: Impact of feline AIM on the susceptibility of cats to renal disease. *Sci Rep* 6: 35251, 2016.

**Figure legends**

**Figure 1. No glomerular lesion was observed in *AIM*$^{-/-}$gddY mice**

**(A)** Representative immunostaining images of IgA (red) and AIM (green) in the renal tissue of 8-week-old gddY, *AIM*$^{-/-}$gddY, and BALB/c mice (n = 5). **(B)** Representative immunostaining images of IgA (red) and AIM (green) in the renal tissue of human patients with IgAN, membranous nephritis (MN), or minimal change nephritic syndrome (MCNS) (n = 4). **(C)** Serum BUN and Cre levels (mg/dL) and urinary protein levels (μg/day) in 8- and 16-week-old gddY and *AIM*$^{-/-}$gddY mice (n = 8, *$p < 0.001$, **$p < 0.01$). All data are expressed as mean ± standard error of the mean and were analyzed using two-way ANOVA. Scale bars: 50 μm (A), 100 μm (B).

**Figure 2. Glomerular IgA deposition did not cause glomerular IgM and IgG co-deposition or complement activation in *AIM*$^{-/-}$gddY mice**

**(A)** Representative immunostaining images of IgA (green), IgG (blue), IgM (red), and C3 (green) in the renal tissue of 16-week-old gddY and *AIM*$^{-/-}$gddY mice (n = 5). **(B)** Average percentage of MAC (C5b-9)-positive area per glomerulus in 8- and 16-week-old gddY and *AIM*$^{-/-}$gddY mice (5 glomeruli were analyzed per mouse (n = 5, $p < 0.05$). Scale bars: 50 μm (A, B)

**Figure 3. Glomerular sclerosis and inflammation were not induced without AIM**

**(A)** Periodic acid–Schiff staining showed less glomerular sclerosis and AZAN staining revealed less glomerular and tubulointerstitial fibrotic changes in 8-week-old *AIM*$^{-/-}$gddY mice. Sclerosis scores were assigned in accordance with the method used in the previous report (15) ($p < 0.05$). Scale bars: 20 μm (PAS), 100 μm (AZAN). **(B)** CD45-positive cells were counted for 15 glomeruli in 8- and 16-week-old gddY and *AIM*$^{-/-}$gddY, and the average number of positive cells per glomerulus was analyzed (n = 5, *$p < 0.01$, **$p < 0.05$. Data are expressed as mean ± standard error of the mean calculated using unpaired two-tailed *t*-test. **(C)** Quantitative PCR analysis for mRNA expression of various inflammatory and fibrotic genes in gddY (white plots) and *AIM*$^{-/-}$gddY (black plots) mice (8- and 16-week-old, n = 3 per group). All data are expressed as mean ± standard error of the mean and were analyzed using unpaired two-tailed t-test (B, C) or two-way ANOVA (D, E).

**Figure 4. rAIM restored glomerular IgM/IgG co-deposition and glomerular damage in *AIM*$^{-/-}$ mice**

**(A)** Representative renal tissue immunostaining images at 2 hours after intravenous administration of 100 μg of rAIM or PBS to *AIM*$^{-/-}$gddY mice (n = 8 per group); IgG (blue),

IgM (red), AIM (green), and C3 (green). Scale bars: 50 μm (upper panel), 20 μm (lower panel). **(B)** (Left) The percentage ratio and percentage of mice with hematuria and proteinuria within 4 hours after the administration of rAIM or PBS. (right) Peak levels of hematuria before rAIM and PBS administration and within 4 hours after administration. All data are expressed as mean ± standard error of the mean. $p < 0.001$; two-way ANOVA.

**Figure 5. AIM was involved in IgA/IgM/IgG immune complex formation in human patients with IgAN**

**(A)** Representative immunostaining images of IgG (blue)/IgM (red)/AIM (green) and C3 (green)/AIM (red) in the renal tissue of human patients with IgAN (n = 4). Furthermore, we assessed whether spontaneous glomerular deposition of AIM or IgM/IgG occurred in the absence of IgA by evaluating *IgA*$^{-/-}$gddY mice. **(B)** Representative immunostaining images of IgG (green), IgM (red), and AIM (green) in the renal tissue of 8-week-old gddY and *IgA*$^{-/-}$gddY mice (n = 5). Scale bars: 50 μm (A), 20 μm (B).

**Figure 6. No immune complex formation of IgA and IgM via AIM was detected in serum samples from gddY mice**

Immunoprecipitation of a serum sample from gddY with anti-IgA (we used the serum of BALB/c and *AIM*$^{-/-}$ gddY as controls) was performed and immune complex was analyzed by immunoblotting with anti-IgM (left), AIM (middle), and IgA (right) under nonreducing conditions.